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The goal of this project is to determine the role of the Src homology 2 domain protein, Grb7, in receptor tyrosine kinase signal transduction. Grb7 is overexpressed in approximately twenty percent of breast cancers coordinately with the HER2 receptor tyrosine kinase. Grb7 binds to HER2 but the role of Grb7 in HER2 signaling is unknown. Our previous attempts to analyze Grb7 function by overexpressing the protein have not been successful. We are unable to detect a phenotype in breast or kidney epithelial cells that overexpress Grb7. We are now generating Grb7 deficient mice as another approach to analyze Grb7 function. We have made good progress in this regard having generated chimeric males containing cells in which one copy of Grb7 has been eliminated. We are currently mating these chimeric males and are generating mice with one copy of Grb7 deleted and mice that do not express Grb7. The mice that do not express Grb7 should provide insight in the role of Grb7 in the normal development of the breast and other organs. They will also be useful in analyzing the effects of decreased Grb7 expression on HER2 induced breast cancer in mice.

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INTRODUCTION

The initial goal of this grant was to determine the function of the Grb7 protein. As previously described, Grb7 is amplified and overexpressed in breast cancer where it is bound to the HER2/Neu protein (1). We have described the close similarity of Grb7 to a gene in *C. elegans* called mig-10 that is involved in neuronal migration (2). Our initial goals were to determine proteins that would lie on the signal transduction pathways with Grb7 and thus would be involved in HER2 signaling. Unfortunately despite repeated efforts we have not been able to demonstrate a binding partner or single pathway upon which Grb7 sits. Our initial failures were based on experiments where we overexpressed Grb7 and looked for abnormal phenotypes in breast cancer or kidney epithelial cells. Despite Grb7 overexpression, no alteration in cell growth or migration could be detected. We have hypothesized that there is sufficient Grb7 in these epithelial cells and increasing the quantities of protein does not affect signaling. Accordingly we have changed strategies and are now generating cells that are deficient in Grb7. To accomplish this we are knocking out Grb7 in mice. To achieve this goal we have made mouse ES cells in which one copy of Grb7 has been deleted. These knockout mice will allow us to study the role of Grb7 in a variety of developmental processes including breast development.

BODY

Methods

I. Identification of Grb7 Targeted ES Cells. A Grb7 targeting vector (pPNTGrb7) was generated as discussed last year (Fig. 1) and was electroporated into mouse Embryonic Stem (ES) cells. Pluripotent ES cells were electroporated using a BioRad electroporator set at 250 mF and 0.3 Kv with an average time constant of 0.3. The pPNTGrb7 used for electroporation was linearized with HindIII and used at a concentration of 20ug pPNTGrb7/0.8 x 10^7 cells. After electroporation, the cells were plated onto neoR Mouse Embryo Fibroblasts (MEF) feeder cells and selected in 300 μg/ml G418 and 0.2 μM Gancyclovir. Colonies were grown for 7-10 days in selection media. Neomycin resistant and Gancyclovir insensitive colonies were isolated and individual clones plated onto 96-well plates. Only clones exhibiting pluripotent morphology were isolated for further culture. Clones were expanded in 96-well plates and frozen stocks and DNA were made from each clone. DNA was harvested from 96-well plates of very densely cultured ES cells. Media was removed, cells rinsed twice with PBS, and lysed overnight at 60°C. Lysis buffer contained 10mM Tris pH 7.5, 10mM EDTA, 10mM NaCl, 0.5% Sarcosyl and 1 mg/ml proteinase K. After lysis, DNA was precipitated using 75mM NaCl/Ethanol at room temperature. The wells were rinsed twice with 70% Ethanol and air dried for 1 hour. DNA was resuspended in Tris EDTA, incubated overnight at 37°C and used for digestion for Southern analysis. DNA was analyzed by Southern blot for homologous recombination using an external probe for Grb 7. Correctly targeted clones were thawed, expanded and rechecked by Southern blottting..

Southern blotting was performed as previously described (1). The DNA was digested overnight with restriction enzyme at 37°C. An additional aliquot of the enzyme

was added in the morning and the DNA was digested for an additional 60 minutes. The DNA was separated on a 0.7% agarose gel, depurinated for 30 minutes, rinsed and denatured two times for 30 minutes and then neutralized three times (3). The DNA was transferred to Nytran overnight in 20X SSC and UV cross linked using 1800 joules. The membrane was air dried and then prehybridized for 1-2 hours at 42°C in 50% formamide, 6X SSC, 5X Denhardts, 1% SDS and 1 mg/ml salmon sperm DNA. The probe was then added, (2 million cpm/ml) in the prehyb buffer and hybridized overnight at 42°. The blots were washed at 0.1X SSC and 0.1% SDS at 42° and then exposed to film.

II. Breeding of Grb7 Chimeras The Transgenic Animal Models Core at the University of Michigan performed the blastocyst injections and subsequent implantations into pseudopregnant females. Chimeras were generated for two cell lines. Both cell lines yielded chimeras with high percentage agouti coat color. The sex ratio of offspring was also skewed toward males. Four males from clone 4B5 and ten males from clone 2E5 (Fig. 3), having greater than 80% agouti coat color, were selected for breeding with C57/BL6 females. Two female mice were placed with a chimeric male for two weeks and then replaced with two new females. In this manner six females in total will be mated with each chimeric male. Using fourteen chimeric males each mating with 6 C57/BL6 females theoretically could yield 84 litters. However for a variety of reasons not all male chimeras will be fertile. The litters obtained are examined for offspring with agouti coat colors. The non agouti mice are euthanized while the agouti offspring will be examined for the presence of the targeted allele by screening for the presence of the Neo allele by PCR.

Results and Discussion

As discussed last year, we have generated a targeting vector in which we removed most of the internal Grb7 sequence (Fig. 1). This targeting vector was designed to generate a Grb7 allele with only a very small fraction of the 5' prime coding region of the gene remaining. This construct was electroporated into 1.6×10^7 ES cells. After selection in gancyclovir and geneticin, 200 colonies which were selected and grown in 96 well plates. From these 200, 150 colonies proliferated such that they could be analyzed by Southern blot. To analyze these clones, DNA from the 96 well plates was prepared as described above and cut with HindIII. We utilized Probe B (Fig. 1) to test the DNA from the electroporated ES cells. Probe B contains sequence that should be present within both the targeted allele as well as the wild type allele. However Grb7 targeted alleles would lose two internal HindIII sites and yield a 15.3 kb band while the wild type allele is 10 kb (Fig. 1). Unfortunately, none of these initial 150 cells had homologous recombination and we proceeded to recheck our targeting vector to ensure it was correct. After further sequencing we confirmed that the targeting vector was correct and proceeded to electroporated 4.4×10^7 ES cells with the Grb7 knockout construct. After isolating 500 clones we were able to screen 480 with analysis by Southern blot as can be seen in Figure 2. By using this technique we were able to obtain three homologous recombinants.

Three positive clones were obtained, 2E5, 4B5 and 5D1. These clones were expanded and two different probes were used to confirm the positive clones. An exon 1 probe (Probe A, Fig. 1) confirmed correct targeting of the 5' end of the recombinant allele. The exon 1 probe showed a 15.3 kb recombinant band and an 8 kb wild type band with a

HindIII digest of genomic DNA. The 3' probe (Probe B, Fig. 1) showed a 15.3 kb recombinant band and a 10 kb wild type band with HindIII digestion, showing correct targeting of the 3' arm of the Grb 7 knock out vector. An exon 3 probe to Grb 7 confirmed the absence of that region in the homologous recombinant clones. The wild type 8kb band is present in all clones and the 15.3 kb band is absent in all clones. The clones were also scored for chromosome counts, with clones having 40 chromosomes being selected for blastocyst injection.

Two clones contained the proper chromosome counts, 4B5 and 2E5, were expanded for injection into C57BL/6 blastocysts. Sixty-five blastocysts were injected for each clone. The ES cells are agouti and X:Y and thus some of the resulting animals should be partially agouti and males should predominate over females. For clone 4B, 36 pups were born of which 20 survived. Eleven of these pups were completely black indicating they were not populated by the targeted ES cells. Of the 9 chimeric mice, 5 were male and between 10% and 90% agouti. For the 2E clone, 29 pups survived of which 6 were black. Of the remaining23, 17 were males and had between 20% and 95% agouti coat color.

Agouti males were grown to 6 weeks and 4 of the 4B and 10 of the 2E chimeric males were mated with C57BL/6 females as described in the methods. From this breeding, agouti offspring should arise indicating that the targeted ES have been incorporated into the male germ line. From the males containing the 4B ES cells, 13 mice have been obtained of which 4 are agouti. From the males containing the 2E ES cells, 29 pups were born of which 8 are agouti. These agouti offspring will have either have received a wild type or Grb7 targeted allele and thus will need to be screened for the presence of the targeted Grb7 allele. This will be performed by looking for the Neo gene (a marker of the targeted allele) by PCR. The mice with the Neo gene will then be confirmed to be heterozygote for Grb7. These Grb7 heterozygotes can then be mated to produce homozygous mice that will not express Grb7. Mice can be confirmed to be homozygote for Grb7 by southern and western blot.

CONCLUSIONS

As discussed in last years report, our present goal is to generate a Grb7 deficient mice. The reasons to depart from the original Statement of Work has been well documented in previous progress reports. Nonetheless despite this departure we feel that we have the same ultimate goal which is to determine the function of Grb7 and understand its role in the function of the breast and other organs. Due to the time required for growth and screening of ES cells as well as the time it takes to perform mouse breeding, the work has not been as extensive as in previous years. Nonetheless we appear to be successful in obtaining heterozgote mice as we have obtained several agouti offspring from our Grb7 chimeric males. We have received a one year no cost extension for our project and this extension will allow us to analyze our heterozygote and homozygote mice.

Our future plans are to breed the heterozygote mice and to examine if homozygote Grb7 mice can be obtained. The exact future plans depend on the phenotype obtained in the Grb7 deficient mice and thus it is difficult to predict the exact experiments in the coming year. The phenotypes could extend from embryonic lethal to completely normal mice. If the mice have obvious defects, it will be necessary to try to characterize the

reasons for the defects and the organs affected. If the mice are phenotypically normal we will need to breed the homozygous animals to ensure they are fertile. It will also be important to determine if the Grb7 deficient mice can feed their pups normally. Mutations within growth factor receptors and their downstraem partners has lead to defects in lactation (4-6) and it is possible that Grb7 is important in this aspect of growth factor receptor signaling. We are confident the final year of the grant will lead to exciting insights into Grb7 function.

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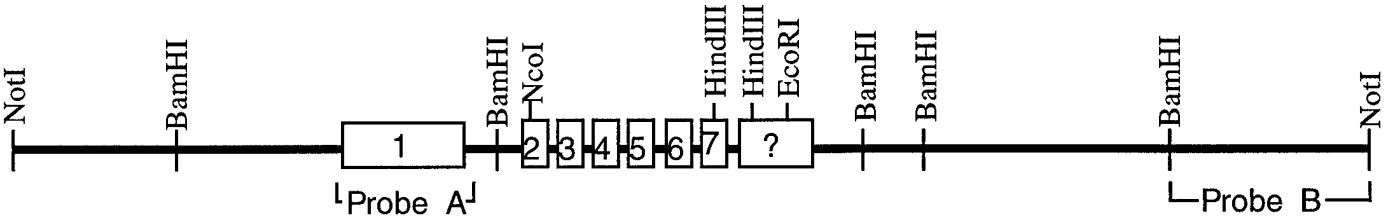
APPENDIX

Figure Legends

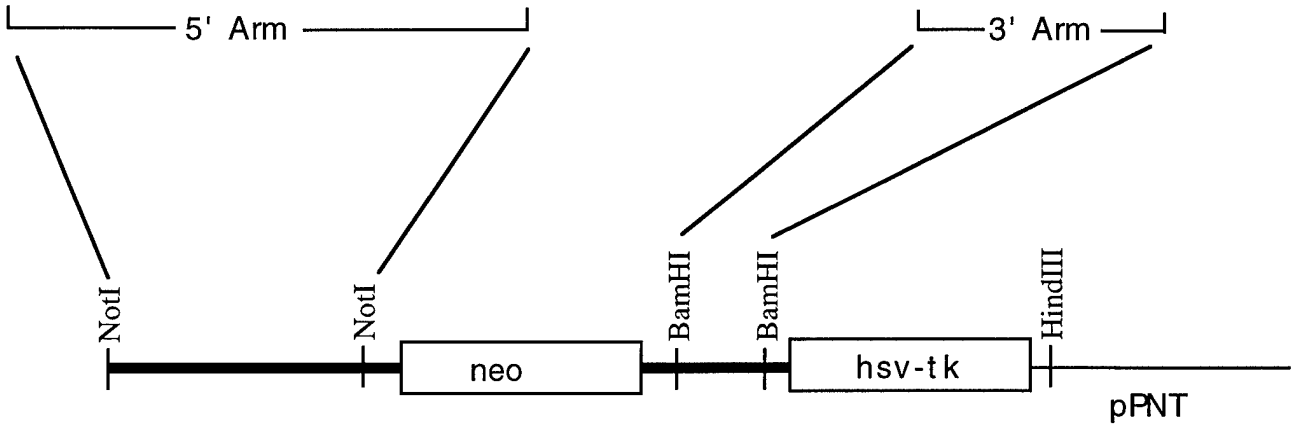
Figure 1. The Grb7 targeted allele. The wild type Grb7 genomic organization was mapped last year. The targeting vector pPNTGrb7 will recombine with the wild type Grb7 allele via its 5' and 3' arms. The targeting vector was electroporated into mouse ES cells to create the Targeted Grb7 allele shown at the bottom of the Figure. The resulting correctly targeted allele will not contain the thymidine kinase gene (hsv-tk) making it resistant to gangyclovir but will contain the Neo gene making it resistant to geneticin. The targeted Grb7 allele will be missing internal HindIII sites leading to different sized fragments when HindIII digested DNA is hybridized with Probe A or Probe B in Southern blotting.

Figure 2. Southern Blotting of ES cells. After electroporation with the pPNTGrb7 targeting vector, ES cells were selected with gangyclovir and geneticin. After selection and growth in 96 well plates, genomic DNA was prepared and cut with HindIII. The DNA was separated on an agarose gel, transferred to nylon membrane and incubated with Probe B (Fig. 1). Most of the ES cells display only one band which represents the wild type allele. One clone indicated with an asterix contains the larger targeted allele. The equal intensity of the wild type and targeted allele confirms the heterozygote genotype in this cell. Other lanes show doublets but the intensity of the different bands are not equal indicating likely artifact due to incomplete digestion of the DNA.

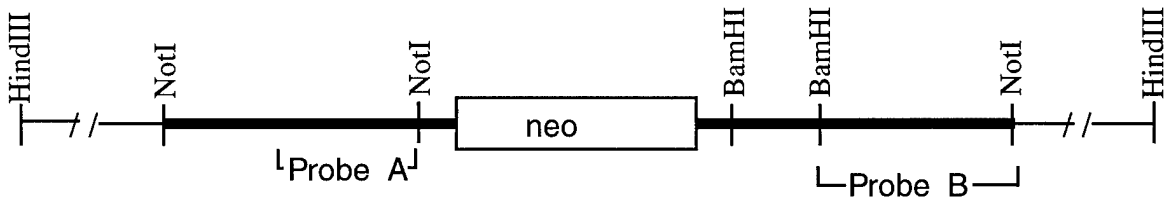
Figure 3. Targeted ES cells. Three clones, 2E5, 4B5 and 5D1, were found to have the targeted Grb7 allele. Using either Probe A or Probe B, the larger Grb7 targeted allele was detected in HindIII digested ES cell DNA. Wild type ES cells or Mouse Embryo Fibroblast (MEF) did not contain the targeted allele. As a control we probed the DNA with a probe from Exon 3 in Grb7 that should be absent in the targeted allele (Fig. 1). As expected this exon is present in the wild type allele but removed from the Grb7 targeted allele.



Wild Type Allele



Targeting Vector-pPNTGrb7



Targeted Grb7 Allele

Figure 1

Southern Screen of ES Cell Clones

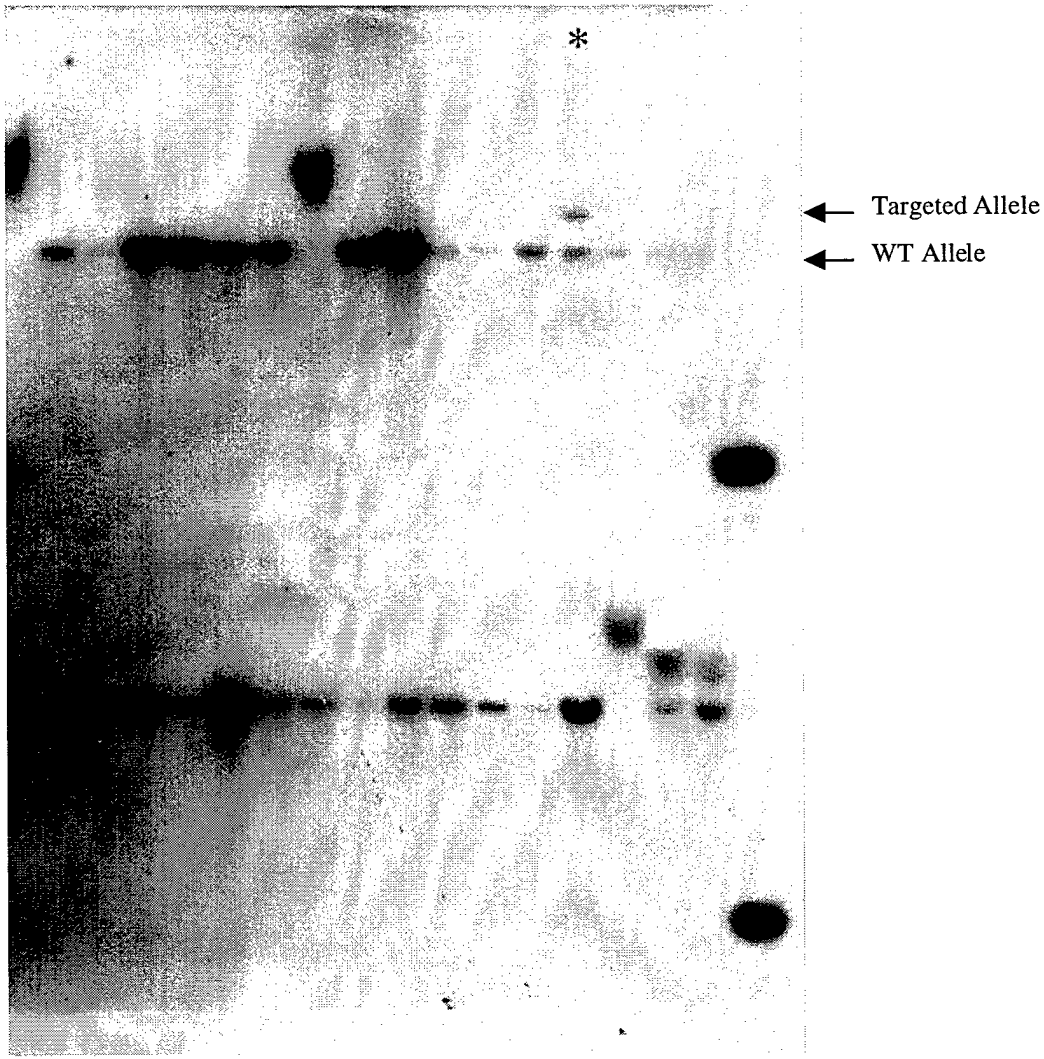


Figure 2

Grb 7 Targeted Clones

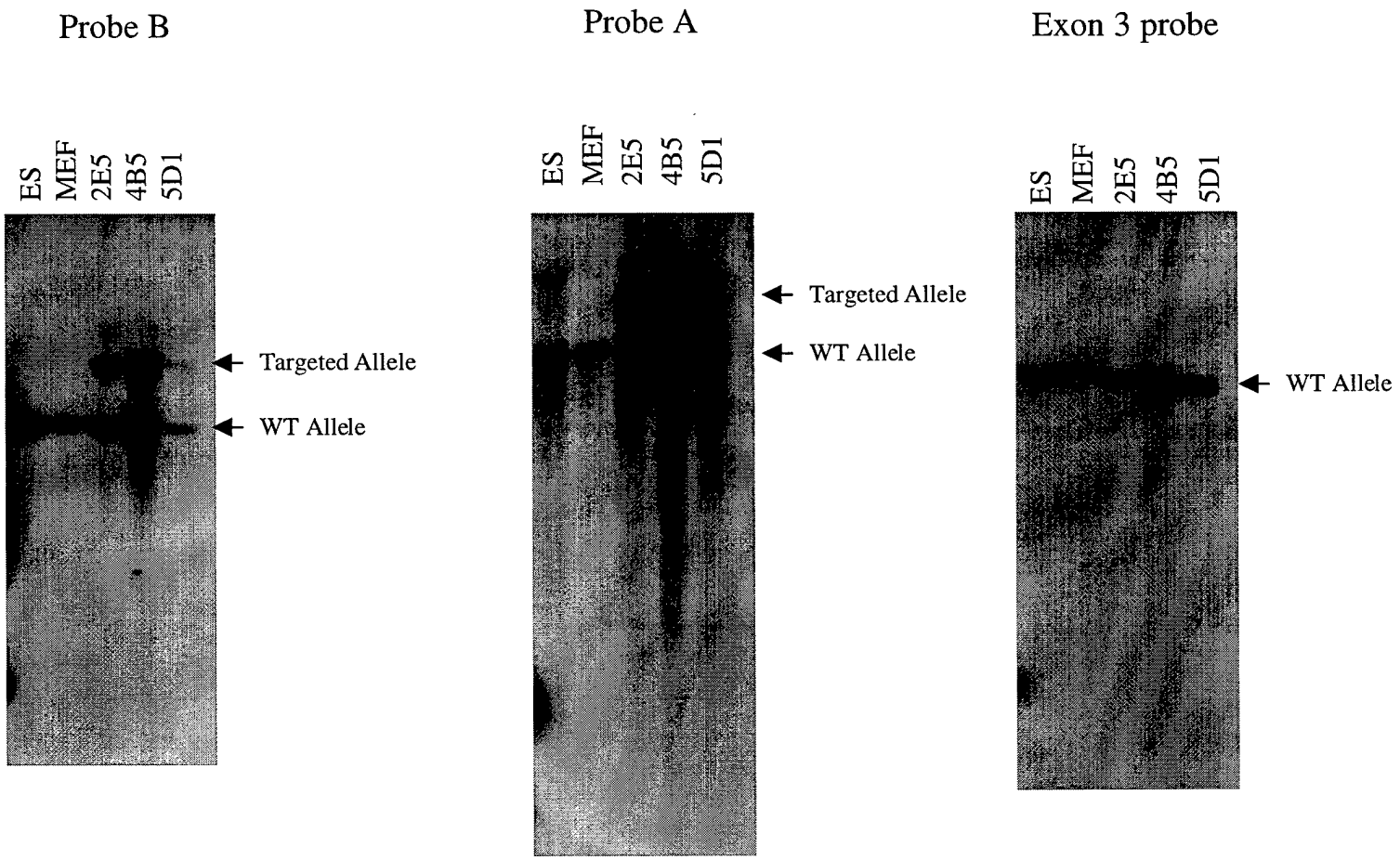


Figure 3